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Qualitative and quantitative phytochemical analysis and antioxidant activities of *Aphanomixic polystachya* leaf

Dipan Sarma and Badal Kumar Datta

Abstract
In this study, *Aphanomixic polystachya* leaves were used to quantify secondary metabolites and evaluated for *in vitro* antioxidant activity. The higher phenolic constituents were estimated in the aqueous extract (14.8±0.2 mg GAE/g) than in the methanol and acetone extracts. The methanol extract contained more tannins (6.81±0.14 mg GAE/g) and higher flavonoid content (15.45±0.1mg RE/g), whereas the acetone extract contained higher saponin (25.72±0.04 mg DE/g). The estimated vitamin C was found to be 3.76±0.05 mg AAE/g. Methanol extract exhibits robust DPPH scavenging activity with an IC_{50} value of 57.85±1.73 µg/mL, and all extract exhibits moderate H_2O_2 radical scavenging activity: methanol (108.08±1.88 µg/mL), aqueous (177.24±4.35 µg/mL), and acetone (224.60±3.66 µg/mL). Acetone extract showed the highest chelating activity with an IC_{50} value (702.06±2.16 µg/mL). The total antioxidant capacity was higher in the acetone extract (37.09±1.04 µg/mL), whereas the aqueous extract exhibited a higher reducing power. These findings suggested that the antioxidant activities in the different *in vitro* systems might be due to the presence of secondary metabolites and vitamin C in the *A. polystachya* leaves extract.

Keywords: *Aphanomixic polystachya;* Secondary metabolites; DPPH; Metal chelating; Total antioxidant capacity; Reducing power activity

1. Introduction
Phytochemicals (Greek: *phyton* = "plant") are naturally occurring chemical substances found in plants that have either beneficial or adverse health effects [1]. Medicinal plants are the richest biorepositories of phytochemical diversity, representing 45% of phenolics, terpenoids, and steroids (27%) and alkaloids (18%) as the most abundant phytochemical groups. These groups of phytochemicals are used to estimate the medicinal properties of plants [2, 3]. In addition, plants contain many structurally diverse phytochemicals derived from primary metabolites, which include carbohydrates, proteins, fats, and secondary metabolites [4]. Secondary metabolites, including alkaloids, phenolics, flavonoids, saponins, tannins, terpenes, glycosides, steroids, and other essential phytochemicals, are distributed throughout various parts of plants. Most studies conclude that the pharmacological activities of medicinal plants are due to secondary metabolites [5, 6]. Secondary metabolites have been reported for biological properties such as antioxidant, anticancer, antibacterial, analgesic, anti-inflammatory, antitumor, antiviral activities, stimulation of the immune system, decrease in platelet aggregation, and modulation of detoxification enzymes and hormone metabolism, and many other activities to a greater or lesser extent [7]. The beneficial therapeutic effects of plant materials usually result from a combination of these secondary metabolites [8]. Moreover, these secondary metabolites used by plants to defend themselves against biotic and abiotic stresses have been converted into medications that may treat various diseases [1, 9].
Antioxidants are chemical compounds that can delay or slow the rate of lipid oxidation reactions, prevent or slow down a target molecule from oxidative damage, directly or indirectly scavenge ROS, or inhibit ROS production [10, 11]. Antioxidants counteract the harmful effects of free radicals by donating electrons or hydrogen atoms to stabilize reactive species, thereby neutralizing their damaging effects. The most common reactive oxygen species (ROS) include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and reactive hydroxyl (OH^-) radicals. The nitrogen-derived free radicals are nitric oxide (NO) and peroxynitrite anion (ONOO) [12]. The detrimental effects of free radicals and other oxidants are counteracted by the natural enzymatic and non-enzymatic antioxidant

defenses of the human
body's complex system.
These free radicals are causing a variety of diseases such as cancer, neural disorders, cardiovascular disease, mild cognitive impairment, Alzheimer's disease, alcohol-induced liver disease, Parkinson's disease, ulcerative colitis, aging and atherosclerosis [9, 13-15]. Substantial evidence suggests that foods containing antioxidants, particularly antioxidant nutrients, may play a crucial role in disease prevention [16]. However, antioxidants can neutralize these free radicals endogenously and exogenously in our bodies [17, 18]. Several plant components, especially secondary metabolites, safeguard cells from free radical damage. Phenols and flavonoids are primarily the main components of plants with therapeutic value and antioxidant effects. Due to their structural characteristics, these components can scavenge these free radicals [19]. The human body counteracts the detrimental effects of free radicals and other oxidants through natural enzymatic and non-enzymatic antioxidant mediators [20, 21]. Thus, antioxidants can significantly enhance the quality of life by halting or delaying the onset of degenerative diseases and substantially reducing healthcare costs [22]. Scientist recommends that antioxidant combinations, rather than any single one, may be more beneficial in the long run. Natural products have been recognized as a guidepost for developing potential medicinal products to address various illnesses due to their rich combination of phytochemicals. Thus, plant-derived natural resources have been isolated and enhanced to mitigate oxidative stress and develop alternative and beneficial treatment forms [23].
The tree *Aphanomixic polystachya* (Family: Meliaceae) is known as Pithraj and Rohituka in the Indian subcontinent. Ethnomedicinally, leaf decoction is used to treat stomach disorders, and oil is used to remedy skin and liver diseases and blood purification [24-26]. The objective of this study is to determine the preliminary phytochemical screening, estimate the secondary metabolites, and assess the *in vitro* antioxidant activities of the leaf extract of *A. polystachya*.

Materials and Methods

Chemicals and reagents: Ascorbic acid; Aluminium chloride; ferric chloride ($FeCl_3$); Folin-Ciocalteu; potassium persulphate; 2, 2-diphenyl-1-picryl-hydrazine-hydrate (DPPH); trichloroacetic acid (TCA), Sulphuric acid; riboflavin; sodium carbonate (Na_2CO_3); sodium hydroxide (NaOH); sodium nitrite ($NaNO_2$); disodium hydrogen phosphate (Na_2HPO_4) and hydrogen peroxide (H_2O_2), Mercuric chloride, Potassium iodide, Sulphuric acid, Ferric chloride, Gallic acid, Polyvinyl pyrrolidone, Rutin, Vanillin, Diosgenin, Metaphosphoric acid; 2, 6- dichloroindophenol sodium salt hydrate; Potassium ferricyanide [$K_3Fe(CN)_6$]; Sodium dihydrogen phosphate (NaH_2PO_4), and all solvents ethanol (99.8%); Acetone (99.8%); used were of analytical grade and Distilled water (DH_2O) was obtained from State Biotech Hub, Tripura University.

Methodology

Collection and Preparation of Plant Powder: *A. polystachya* leaves were collected from Pandabpur (23°45'59.4"N, 91°14'35.5"E), West Tripura, NE India. The plants *A. polystachya* were made soil and dust-free and clean by washing them with plain water, followed by distilled water. The plant parts were then shade-dried until they could be made into powder. These shade-dried parts were then ground into powder in a mixture grinder.

Preparation of Extracts: The crude extract was prepared from the dried plant powder following the cold percolation method. Extracts of *A. polystachya* leaves are prepared by soaking 15g of powdered leaves in 100 ml of three solvent systems: methanol, acetone, and aqueous, for each respective crude extract. Then the methanol and acetone extracts were evaporated by a Rotary evaporator. The aqueous extract was cooled and dried by a Lyophilizer (Freeze dryer) to concentrate the extract. Crude solvent extracts were used to study the quantification of phytochemicals, antioxidants, and antibacterial activities.

Qualitative phytochemical screening of *A. polystachya* leaf extracts

A) Qualitative Estimation: The qualitative phytochemical screening of alkaloids, flavonoids, phenols, and tannins of plant extract was done by the following methods described by Khalid *et al*. (2018), with slight modification [27].

1. Test for Alkaloid: The plant extracts were mixed in 1% v/v HCL. The mixtures were warmed and filtered. Then, these filtrate extracts were used for the following test.

a. Mayer's test: The filtrate extracts were reacted with Mayer's reagent (1.36 g Mercuric chloride + 5 g Potassium iodide in 100 ml water). The formation of yellow-colored precipitates indicates the existence of alkaloids.

2. Test for Flavonoids: Alkaline reagent test: The plant extracts were reacted with 2-3 drops of sodium hydroxide solution to form an acute yellow color, which changed to colorless by adding some drops of hydrochloric acid. This observation specifies the presence of flavonoids.

3. Test for phenols and tannin: In a test tube, 20 mg of the powder sample of each extract was diluted in 20 ml of distilled water. Adding 3-4 drops of 0.1% v/v Ferric chloride to each sample changed the color to brownish green or blue. This observation indicates the presence of phenols or tannins**.**

4. Test for glycosides: Froth test for saponin glycosides: The plant extract is diluted in distilled water and shaken for 15 minutes in a graduated cylinder. The formation of a one cm layer of foam indicates the presence of saponins.

5. Test for phytosterols: Salkowski's test: The plant extract was mixed with chloroform. Then 5-6 drops of conc. Sulphuric acid was treated with these extracts, shaken gently, and carefully allowed to stand for some time. The golden yellow color indicates the presence of triterpenes (phytosterol).

Quantitative estimation of secondary metabolites

Determination of Total Phenolic Content (TPC): In a test tube, 10 mg of the extract was dissolved in 10 mL of the respective solvent. From it, 30 µg or 30 µL of the extract (each 10 mg of extract dissolved in 10 mL of the respective solvent) was taken in a test tube and diluted to a volume of 1 mL with distilled water. Then, 500 µl of Folin-Ciocalteu reagent (1:10 with water) and 2 ml of 20% Sodium carbonate (Na_2CO_3) solution (20%; w/v) were added sequentially to each tube. The reaction mixture was quickly vortexed, and the tubes were left in the dark for 40 minutes to allow for the

formation of color. Then, the absorbance at 725 nm was measured and compared to the reagent blank. The linearity of the standard gallic acid calibration curve was established in the range of 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL. Using the standard curve, the total phenolic content of the plant extract was calculated and expressed as milligrams of gallic acid equivalent (mg GAE/mg extract) [28].

Determination of Total Tannin Content (TTC): 10 mg of the extracts were taken and dissolved in 10 mL of the extraction solvent in a test tube. In another test tube, 1.0 mL of distilled water was added to 100 mg of polyvinylpyrrolidone. Then, 1 ml of the phenolic extract with tannin was added. For four hours, this solution was incubated at 4 °C. The sample was then centrifuged for 5 minutes at 5000 rpm, and 50 µg or 50 µL of the supernatant was taken. The amount of free phenolics in the supernatant was calculated on a dry matter basis and measured at 725 nm to determine its phenol content. A standard curve was constructed, followed by TPC. From the results, the tannin content of the extract was calculated as follows [29].
Tannins (mg GAE/g extract) =Total phenolics (mg GAE/g) - Free phenolics (mg GAE/g)

Determination of Total Flavonoid Content (TFC): The stock solution of plant extract is prepared in a test tube by dissolving 10 mg of the extract in 10 mL of the extraction solvent. A test tube containing 30 µg or 30 µL of the extract was filled with 150 µL of 5% sodium nitrite solution before being topped off with distilled water to a final volume of 1 mL. After incubating for 5 minutes, 150 µL of a 10% aluminum chloride solution was added, and the mixture was left to stand for 6 minutes. After that, 2 mL of 4% sodium hydroxide solution was added, and the volume was adjusted to 4 mL with distilled water. After thoroughly shaking, the mixture was left at room temperature for 15 minutes. At 510 nm, the absorbance was measured. The pink color's appearance indicated the existence of flavonoid content. A standard calibration curve for rutin was drawn in the same manner as the TPC. Using the standard curve, the total flavonoid content was expressed as Rutin equivalent (mg RE/g extract) on a dry weight basis [30].

Determination of Total Saponin Content (TSC): The vanillin-sulphuric acid colorimetric reaction method was modified to measure the total saponin concentration. To prepare the stock solution of plant extract, 10 mg of the extract is dissolved in 10 mL of the extraction solvent in a test tube. Within 950 µL of distilled water, 50 µL of plant extract was added. An additional 250 µL of the vanillin reagent (800 mg of vanillin in 10 ml of 99.5% ethanol) was also added. Then, 2.5 mL of 72% sulfuric acid was added and thoroughly mixed. This solution was maintained at 60 °C in a water bath for 10 minutes. After cooling for 10 minutes in ice-cold water, the absorbance was measured at 544 nm. A calibration curve of Diosgenin was constructed in the same way as the TPC. The values were expressed as diosgenin equivalents (mg DE/g extract) derived from a standard curve [31].

Determination of Ascorbic acid (Vitamin C): 10 mg of plant powder was reextracted using 10 ml of 3% Metaphosphoric acid. Following 45 minutes of standing time at room temperature, the sample was filtered using Whatman No. 1 filter paper. 9 mL of 2, 6-dichloroindophenol sodium salt hydrate at a concentration of 50 µmol/L was added to 1 mL of the filter. The absorbance was measured at 520 nm after 30 minutes. A calibration curve of ascorbic acid was constructed in the same way as the TPC. Ascorbic acid content was calculated based on the L-ascorbic acid calibration curve. The results were expressed as mg of ascorbic acid equivalent (mg AE/g extract) [32].

In-vitro antioxidant activity:

DPPH free radical scavenging assay: The radical scavenging potential of plant extracts was assessed using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method, as described in the recent method [9]. Briefly, different concentrations (100 to 500 µg/mL) of the plant extract and standard ascorbic acid were added to separate test tubes. After that, 0.5 mL of freshly prepared DPPH (0.5 mM) was added to each sample, thoroughly mixed, and incubated for 30 min in a dark chamber. Afterward, the absorbance was recorded at 517 nm, and the free radical scavenging activity was calculated using the equation of

$$\text{DPPH radical scavenging activity (\%)} = \left[\frac{A_c - A_s}{A_c}\right] \times 100$$

Where Ac is the control absorbance of DPPH radical + methanol; As is the absorbance of DPPH radical + sample AgNPs/standard Ascorbic acid.

Hydrogen peroxide scavenging (H_2O_2) assay: The hydrogen peroxide scavenging ability of plant extracts was assessed using the method described by Ruch *et al*. (1989) [33], which was employed to determine the efficiency of plant extracts in scavenging hydrogen peroxide. A 40 mM hydrogen peroxide solution was produced in 50 mM phosphate buffer (pH 7.4). After adding the extract (100-500 g/mL) to the hydrogen peroxide, distilled water is added. After 10 minutes, the absorbance at 230 nm is compared to a blank phosphate buffer solution without hydrogen peroxide. The following formula was used to compute the percentage of hydrogen peroxide scavenging:

$$\%\ \text{Scavenged } (H_2O_2) = \left[\frac{A_c - A_t}{A_c}\right] \times 100$$

Where A_c is the absorbance of the control, and A_t is the absorbance of the test samples at different concentrations

Reducing power method (RP): RP activity was estimated by the modified method described by Oyaizu (1986) [34]. 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of $K_3Fe(CN)_6$ (1% w/v) are reacted with 0.2 mL of various concentrations of the sample solution (100-500 µg/mL) dissolved in distilled water. After 20 minutes of incubation at 50 ºC, 0.5 mL of 10% w/v trichloroacetic acid was added to the resultant mixture. The mixture was centrifuged at 3000 rpm for 10 minutes, and 0.5 mL of the supernatant from the solution was collected. This supernatant was then mixed with 0.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1%, w/v). The absorbance is then measured at 700 nm against the blank sample.

Metal chelating assay: The metal chelating activity of various solvent extracts from *A. polystachya* leaves was estimated using the method described by Soler-Rivas *et al*. (2000) with modifications [35]. For this test, a solution of 0.5 mL ferrous chloride (0.2 mM) was mixed with 0.1 mL of

various solvent extract concentrations ranging from 100 to 500 µg/mL. The reaction was initiated by adding 0.2 mL of ferrozine (5 mM) and incubating it at room temperature for 10 minutes, followed by measurement of the absorbance at 562 nm. EDTA was used as a positive control. The following formula was used to calculate the percentage of metal chelating activity.

$$\text{Percentage (\%) metal chelating activity} = \frac{[Ac-As]}{Ac} \times 100$$

Where Ac is the absorbance of the control, and As is the absorbance of the samples at different concentrations

Phosphomolybdate assay: Using ascorbic acid as a reference, the total antioxidant capacity (TAC) of each solvent extract was measured using the phosphomolybdate assay technique with slight modifications [36]. In brief, a 0.1 mL aliquot of sample solution was combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were sealed and placed in a water bath at 95°C for 90 minutes. The absorbance of the combination was measured at 765 nm against a blank after the samples had cooled to room temperature. A typical blank was incubated with 1 ml of the reagent solution and the corresponding solvent volume under the same settings. Ascorbic acid was utilized as a control.

$$\text{Percentage (\%) TAC} = \frac{[Ac-As]}{Ac} \times 100$$

Where Ac is the absorbance of the control, and As is the absorbance of the samples at different concentrations

Statistical analysis: The mean±standard deviation of triplicate data (n=3) is calculated in MS Excel 2019. All analyses of ANOVA and Tukey's multiple comparison tests were carried out using the statistical software program R 3.6.2 (R Core Development Team, 2019) [37].

Results and Discussion

Qualitative phytochemical screening of *A. polystachya* leaf extracts

Phytochemical screening of crude extracts of different solvents of five selected medicinal plants demonstrated the presence of alkaloids, phenol, tannin, flavonoids, saponins, and Phytosterol, as shown in Table No.1. Based on the qualitative screening of phytochemicals, the quantitative estimation was carried out.

Table 1: Qualitative phytochemical screening of various solvent extracts of *A. polystachya* leaf

A. polystachya leaf	Alkaloid	Phenol & Tannins	Flavonoid	Saponin	Phytosterol
Aqueous extract	+++	+	+	+++	+++
Methanol extract	++	+++	+++	+	++
Acetone extract	+	++	++	++	++

Quantitative estimation of secondary metabolites: Quantitative estimation of secondary metabolites from *A. polystachya* leaf extract is presented in Table 2.

Total Phenolics Content: The total phenolic contents (TPC) of methanol, acetone, and aqueous extract of different parts of *A. polystachya* varied widely (Table 2). The total phenolic content of A. *polystachya* leaf aqueous extract amounted to 14.8 mg GAE/g, followed by methanol extract at 8.35 mg GAE/g, whereas A. *polystachya* leaf acetone extract amounted to 4.81 mg GAE/g.

Estimation of Total Tannin Content: The methanol extract of *A. polystachya* showed a higher tannin content (6.81 mg GAE/g), followed by the acetone extract (3.23 mg GAE/g) and the aqueous extract (1.47 mg GAE/g), as represented in Table 2.

Table 2: Quantitative determination of the chemical constituency of different solvent extracts of *A. polystachya* leaf

A. polystachya leaf	Total Phenol (mg GAE/g)	Total Tannin (mg GAE/g)	Flavonoid (mg RE/g)	Saponin (mg DE/g)
Aqueous extract	14.8±0.2	1.47±0.07	15.45±0.1	9.94±0.05
Methanol extract	8.35±0.1	6.81±0.14	12.1±0.14	10.43±0.01
Acetone extract	4.81±0.06	3.23±0.2	5.25±0.14	25.72±0.04

Total Flavonoid Content: The total flavonoid contents (TFC) of the *A. polystachya* plant are tabulated in Table No. 2. The TFC of *A. polystachya* varies from 5.25 to 15.45 mg RE/g. It was found that aqueous extract (15.45 mg RE/g) showed a higher TFC followed by methanol (12.1 mg RE/g) and acetone extract (5.25 mg RE/g).

Estimation of Total Saponin Content: The total saponin contents (TSC) of the various extract of different parts of *A. polystachya* plant was estimated based on the vanillin - sulphuric acid colorimetric reaction and is tabulated in Table No. 2. The TSC of acetone *A. polystachya* leaf extract was recorded at 25.72 mg DE/g, followed by methanol and aqueous extract (10.43 and 9.94 mg DE/g) respectively.

Estimation of Ascorbic acid (Vitamin C): The ascorbic acid content of *A. polystachya* leaves was estimated to be 3.76 mg AAE/g.

In vitro Antioxidant Activities

The natural sources of antioxidants, such as dietary antioxidant intakes from foods and medicinal plants, enhance the antioxidative capacity of the plasma and promote the normal functioning of physiological systems [38, 39]. Several techniques have been studied to determine the antioxidant activity *in vitro*, allowing for the rapid screening of substances.

In this study, the antioxidant activity of *A. polystachya* leaf was evaluated using the DPPH free radical scavenging method, Hydrogen peroxide scavenging (H_2O_2), reducing power method, Metal chelating activity, and Total Antioxidant Capacity Assays. The results were expressed as IC_{50}, which means the sample concentration required to decrease the absorbance by 50%. A more substantial antioxidant activity is characterized by a lower IC_{50} value, whereas a higher IC_{50} value indicates less significant scavenging activity [40]. Thus, the higher % RSA (radical

scavenging assay) and lower IC_{50} values indicate a higher antioxidant activity. IC_{50} values were determined using regression equations derived from extract concentrations and percentage inhibition of different methods of antioxidant assay, e.g., DPPH assay, H_2O_2 radical scavenging assay, Metal chelating assay, and phosphomolybdate assay.

DPPH free radical scavenging assay

DPPH acts as a stable free radical. Antioxidants neutralized the free radical property of DPPH through interaction, either by transferring electrons or hydrogen atoms to DPPH [41, 42], which changed its color from purple to yellow in reaction with test samples and decreased its wavelength at 517nm [9.42]. The results on the percentage radical scavenging activity of *A. polystachya* leaves in various extracts and best-known antioxidant standards, viz., ascorbic acid and BHA, at different concentrations are presented in Figure 1. *A. polystachya* leaves extracts demonstrated a good DPPH scavenging activity. The methanolic extract (APM) shows the highest scavenging activity (89.2±0.074%) at the concentration of 100 µg/mL. The DPPH scavenging activity of acetonic (APA) and aqueous (APW) extracts was 95.83±0.044% and 88.84±0.22%, respectively, at a concentration of 500 µg/mL, whereas APM showed 87.51±0.66% at the same concentration. APA exhibits the highest %DPPH scavenging activity. The calculated IC_{50} of methanolic, acetonic, and aqueous extracts was 57.85±1.73, 152.25±1.08, and 70.55±0.56 µg/mL, respectively. These results suggested that the methanol extract of *A. polystachya* leaves exhibits higher scavenging activity with lower IC_{50} values (57.85 µg/mL), followed by the aqueous extract (70.55 µg/mL) and the acetone extract (152.25 µg/mL), respectively. DPPH scavenging activity of *A. polystachya* leaves, various solvent extracts, along with standard antioxidant agents, ranked in the following order: Ascorbic acid> BHA> APM> APW> APA.

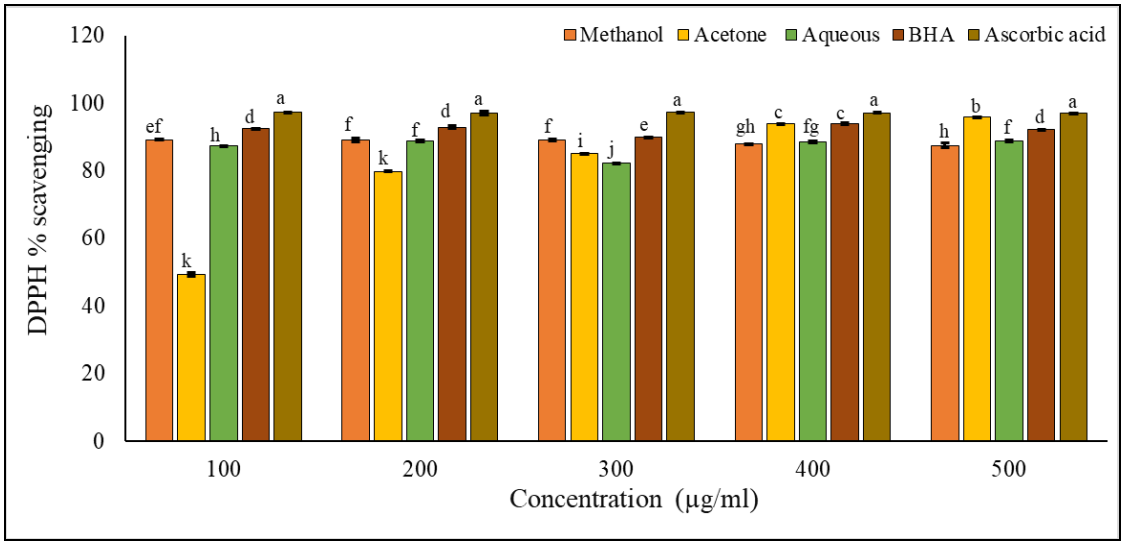

Fig.1. DPPH radical scavenging activity of different extracts of *A. polystachya* leaves by different solvents at various concentrations. %DPPH scavenging with the same letter on bars is not significantly different at P ≤0.05 by Tukey's multiple comparison test.

Table 3: Radical scavenging activities of different solvent extracts of *A. polystachya* leaf at different concentrations

A. polystachya leaf	IC_{50} values(µg/mL) of radical scavenging			
	DPPH radical scavenging	H_2O_2 radical scavenging	% Metal chelating	% TAC
Aqueous extract	70.55±0.56	177.24±4.35	3904.70±5.86	57.06±1.51
Methanol extract	57.85±1.73	108.08±1.88	3065.83±6.63	50.23±1.06
Acetone extract	152.25±1.08	224.60±3.66	702.06±2.16	37.09±1.04
Ascorbic acid	26.22±0.24	16.92±1.22	-	11.3±1.41
BHA	46.63±0.3	-	-	-
EDTA	-	-	206.2±0.11	-

Hydrogen peroxide scavenging (H_2O_2) assay: Hydrogen peroxide (H_2O_2) can enter the body through contact with the eyes, skin, or inhalation of vapor or mist. The rapid breakdown of H_2O_2 into oxygen and water can result in the formation of hydroxyl radicals (OH^-), which are highly reactive and short-lived. These highly reactive hydroxyl radicals (OH^-) can initiate lipid peroxidation, resulting in significant damage to cells, their DNA, and other bodily components [15, 43]. Therefore, removing hydroxyl radicals, which have a detrimental effect on our bodies, is crucial. Figure 2 represents the % H_2O_2 scavenging activity of various solvent extracts of *A. polystachya* leaves at different concentrations. Methanolic extract of *A. polystachya* leaves (APM) demonstrated ~70-91% H_2O_2 scavenging activity at various concentrations. At a concentration of 400 µg/mL, the highest scavenging activity of APM was 91.31±1.04%. Acetonic extract of *A. polystachya* leaves (APA) exhibited the highest scavenging activity of 95.3±0.34% at a concentration of 200 µg/mL. It was assessed that APA gradually decreased its scavenging activity at concentrations of 300, 400, and 500 µg/mL, with the percentage of H_2O_2 scavenging activity being 68.08±0.87%, 49.32±0.76%, and 40.95±0.76%, respectively. Aqueous extract of *A. polystachya* leaves (APW) scavenges ~38-88% of H_2O_2 at various concentrations. However, the highest H_2O_2 scavenging activity for APW was 87.93±0.77% at a concentration of 100 µg/mL. APA showed the highest % H_2O_2 scavenging activity among APM and APW. The estimated IC_{50} of APM, APA, and APW was 108.08±1.88, 224.60±3.66, and 177.24±4.35 µg/mL. The ranking orders of H_2O_2 scavenging activities of various solvent extracts of *A. polystachya* leaves are as follows: Ascorbic acid> APM>APW> APA. Among the solvent extracts, methanol

exhibited the lowest IC_{50} value (108.08±1.88 µg/mL), indicating a strong H_2O_2 scavenging activity.

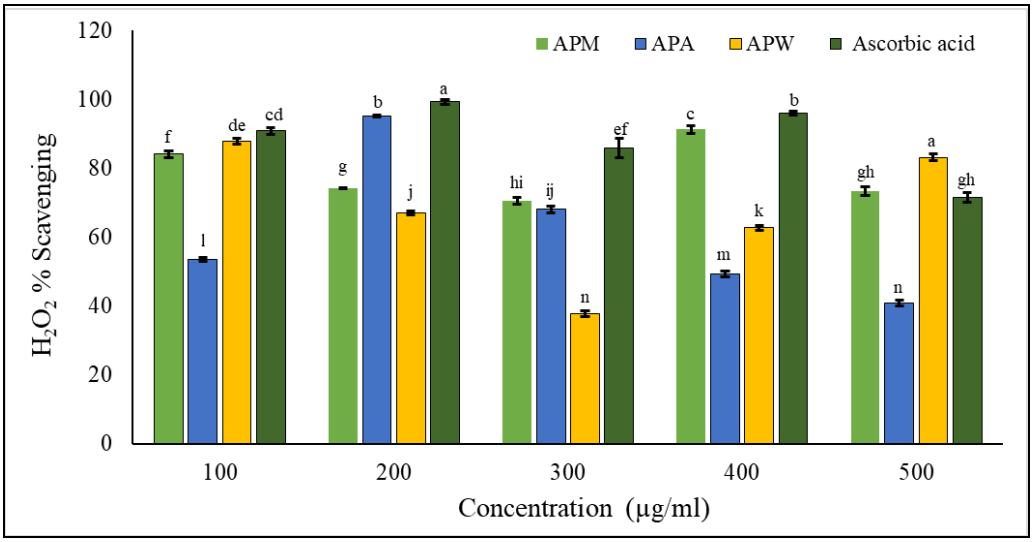

Fig.2. H_2O_2 radical scavenging activity of different extracts of *A. polystachya* leaves by different solvents at various concentrations. Each value represents a mean±SD (n = 3). % H_2O_2 scavenging with the same letter on bars is not significantly different at P ≤0.05 by Tukey's multiple comparison test.

Reducing power method (RP): The reducing power technique is based on the principle of increasing the absorbance of the reaction mixtures. An increased absorbance is an indication of higher antioxidant activity. In this method, the antioxidant compounds combine with potassium ferricyanide, trichloroacetic acid, and ferric chloride to form a colored complex, which is measured at 700 nm. The increased absorbance of the reaction mixture indicates that the samples possess reducing power [44, 45]. The increase in absorbance values with increasing concentrations is presented in Table 4. The reducing power activity of various solvent extracts of *A. polystachya* leaves at different concentrations, with significant differences among the extracts, is illustrated in Figure 3. The estimated rank of the reducing power activity is aqueous extract> methanol extract>acetone extract. The reducing power ability increased with the increasing concentration of solvent extract due to a higher concentration of secondary metabolites in each extract concentration.

APM and APW showed a higher reducing power activity than APA. The maximum absorbance suggested a higher reducing power activity. It was found that APW exhibits maximal absorbance at 700 nm, indicating the highest reducing power activity. The reducing power exhibits a good linear relationship (R^2 > 0.9) in these three extracts (Table 4). indicates that reducing power is concentration-dependent and increases with increasing concentrations.

Table 4: Absorbance of various solvent extracts of *A. polystachya* leaves at various concentrations (µg/mL) in the ferric reducing power determination model

Concentration	Abs. of APM	R^2 of APM	Abs. of APA	R^2 of APA	Abs. of APW	R^2 of APW
100 (µg/mL)	0.24±0.0002	0.9157	0.077±0.001	0.9855	0.281±0.001	0.9649
200 (µg/mL)	0.266±0.003		0.143±0.001		0.348±0.001	
300 (µg/mL)	0.327±0.003		0.192±0.002		0.501±0.002	
400 (µg/mL)	0.392±0.001		0.319±0.002		0.661±0.002	
500 (µg/mL)	0.571±0.006		0.388±0.004		0.729±0.032	

Values are mean±SD of three parallel measurements

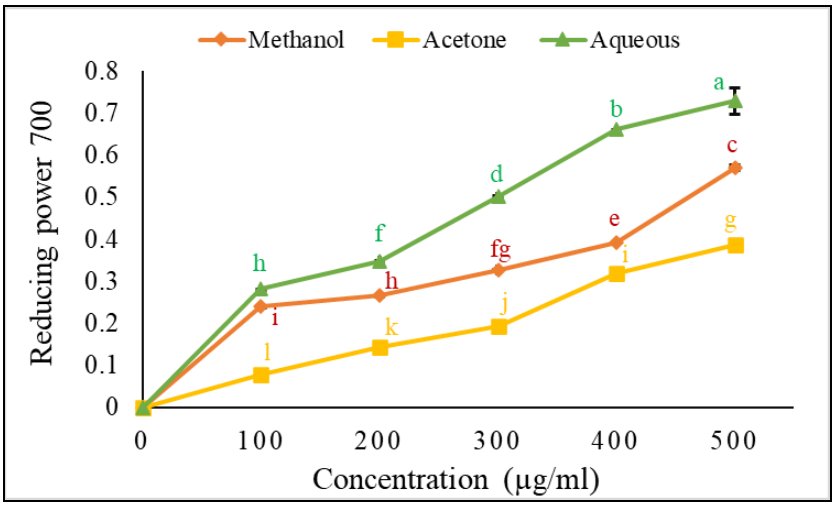

Fig.3. Reducing power activity of different extracts of *A. polystachya* leaves by different solvents at various concentrations. Each value represents a mean±SD (n = 3). Absorption at 700 nm on the RP model with the same letter on bars is not significantly different at P ≤0.05 by

Tukey's multiple comparison test.

Metal chelating activity: Ferrozine can form a complex with a red color by forming chelates with Fe^{2+}. This reaction is inhibited in the presence of other chelating agents, resulting in a decrease in the red color of the ferrozine-Fe^{2+} complexes. Measurement of the color reduction determines the chelating activity, which competes with ferrozine for ferrous ions [35]. The ferrozine-Fe^{2+} complexes, characterized by a reduced red color, indicate that the samples exhibit significant scavenging activity [46].

In this study, Fig. 4 represents the % metal chelating activity of solvent extracts of *A. polystachya* leaves and standard EDTA. Acetone extract exhibits higher chelating activity (34.95±0.1) at a 500 µg/mL concentration compared to methanol and aqueous extracts. The IC_{50} of metal chelating activity was evaluated, which refers to the concentration of the sample required to decrease the absorbance of Ferrozine by 50%. This value is tabulated in Table 3. The acetone extract exhibits the lowest IC_{50} value (702.06±2.16 µg/mL), followed by the methanol extract (3065.83±6.63 µg/mL) and the aqueous extract (3904.70±5.86 µg/mL). The effective chelating ability was found to be in the order of acetone extract > methanol extract> aqueous extract. The standard chelating agent EDTA has an IC_{50} value of 206.2±0.11 µg/mL.

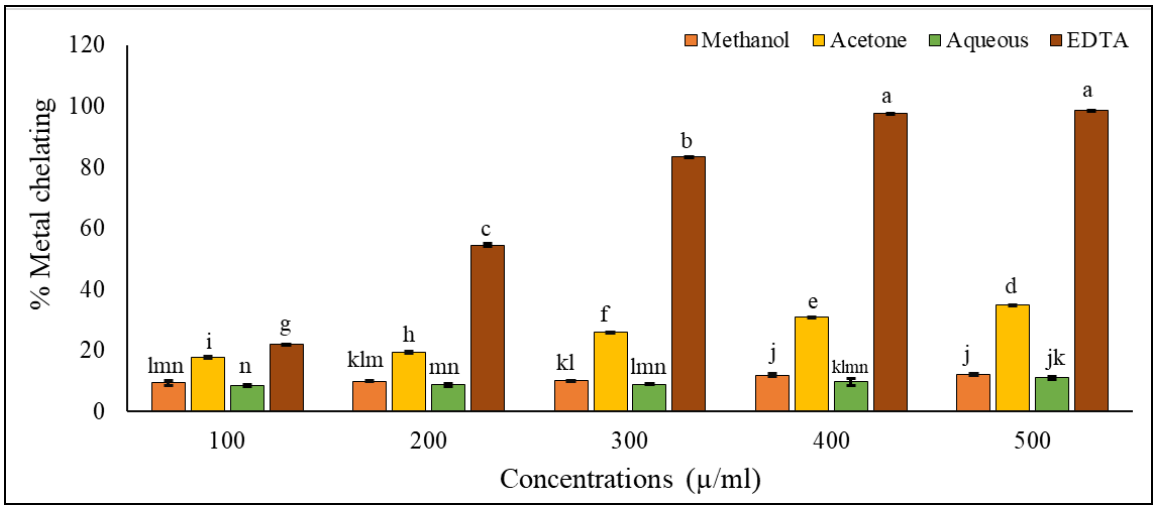

Fig.4. Metal chelating activity of different extracts of *A. polystachya* leaves by different solvents at various concentrations. Each value represents a mean±SD (n = 3). % Metal chelating activity with the same letter on bars is not significantly different at P ≤0.05 by Tukey's multiple comparison test. Each value represents a mean±SD (n = 3).

Phosphomolybdate assay: The phosphomolybdate assay is a quantitative estimation based on the reduction of the phosphomolybdate ion, which is determined spectrophotometrically with a maximum absorption at 765 nm [47]. Since the total antioxidant capacity (TAC) is expressed as ascorbic acid equivalent [48], this method is used. In this assay, Mo(VI) is reduced to Mo(V) by the test sample reaction and subsequently forms green phosphate/Mo(V) compounds in the presence of antioxidant compounds, which are measured spectrophotometrically with a maximum absorption at 765 nm [43]. The total antioxidant scavenging (%TAC) decreased in the order of methanol extract>acetone extract> aqueous extract. At the highest concentration of each solvent extract (500 µg/mL), the acetone extract showed the most increased antioxidant activity (86.64±0.95 µg equivalents of ascorbic acid), followed by aqueous extract (74.04±0.51 µg equivalents of ascorbic acid) and methanol extract (54.57±1.16 µg equivalents of ascorbic acid).

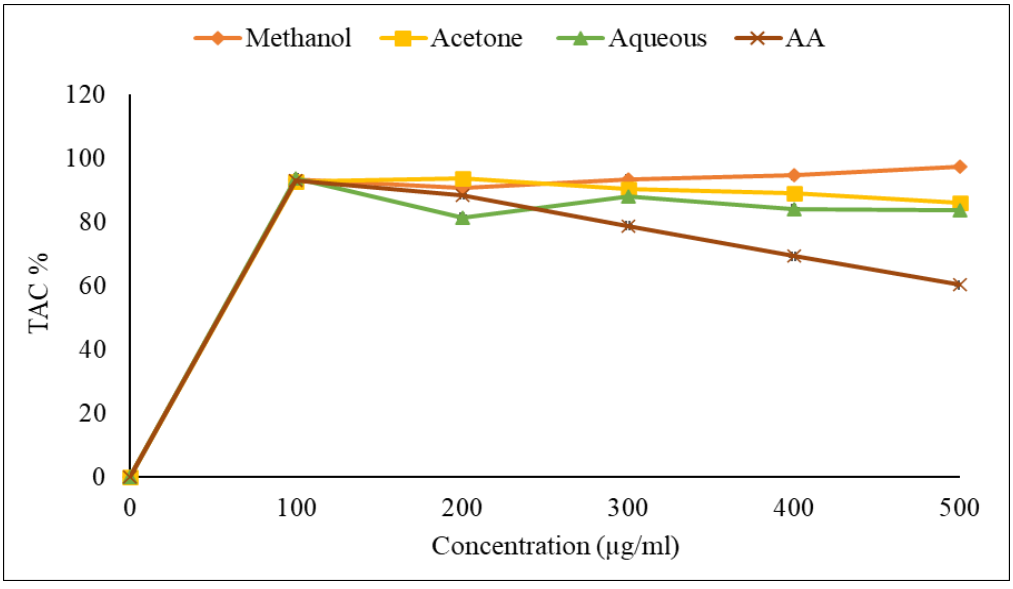

Fig.5. Phosphomolybdate assay activity of different extracts of *A. polystachya* leaves by different solvents at various concentrations. Each value

represents a mean±SD (n = 3).

The secondary metabolites, primarily phenolic and flavonoid compounds, have been identified as free radical scavengers and are considered promising therapeutic agents for diseases related to free radicals [49, 50]. Several research studies have demonstrated that plant saponins exhibit robust antioxidant activity. Thus, saponins might represent new prospective candidates for antioxidants due to their capacity to neutralize free radicals; they may also represent new prospective candidates for antioxidants, depending on their ability to scavenge free radicals [51, 52]. Water-soluble polyphenols, specifically tannins, are found in plants and are recognized as a natural source of antioxidants. It has been reported that polyphenols and tannins exhibit protective activity against DNA damage by inhibiting the generation of superoxide radicals [42, 53]. However, tannins also cause toxic effects by promoting the formation of DNA strand breaks, thereby decreasing cellular viability [54]. Vitamin C in humans is a potent water-soluble antioxidant and acts as an electron donor [55]. According to epidemiological research, individuals who consume large amounts of vitamin C have a lower risk of developing various chronic diseases, including heart disease, cancer, eye disorders, and neurological problems [42]. The solvent extract comprises various phytochemicals, featuring a balanced composition of phenolics, tannins, flavonoids, and saponins. Ascorbic acids likely provide synergistic antioxidant action through both hydrogen atom transfer and single electron transfer mechanisms. The solvent extract comprises various phytochemicals, featuring a balanced composition of phenolics, tannins, flavonoids, saponins, and Ascorbic acids, likely providing synergistic activity through both hydrogen atom transfer and single electron transfer mechanisms, which enhance and boost the antioxidant potential [45] (Siddeeg *et al*., 2021)

Conclusion and Future Perspective

This study found that all crude solvent extracts, i.e., methanol, acetone, and aqueous extract, contain secondary metabolites and demonstrated free radical scavenging activity, reducing power, and chelating activity. These activities may include phenolic and flavonoid compounds in *A. polystachya* leaves, as well as various solvent extracts. Methanol extract demonstrated maximum antioxidant activity through DPPH and H_2O_2 free radical scavenging, whereas acetone extract showed maximum chelating activity. A higher total antioxidant capacity was observed in the acetone extract. The mechanism of action of the antioxidant activities in different *in vitro* systems of *A. polystachya* leaves crude extract may be due to the action of individual phytochemicals or the synergistic action of multiple phytochemicals, such as polyphenolic and flavonoid compounds, which contribute to the neutralization of radicals. Our study suggested that extracts of *A. polystachya* leaves possess antioxidant potential and could be used as a natural source of antioxidant agents against degenerative diseases induced by free radicals. Future research should focus on bioassay-guided fractionation and chromatographic isolation to identify and structurally characterize the specific phytoconstituents responsible for the antioxidant activities. Moreover, the active molecules in these crude extracts need to be identified using advanced analytical tools, such as LC-MS/MS, NMR spectroscopy, and HPLC-DAD profiling, which can be employed to elucidate the chemical fingerprints of bioactive fractions responsible for their *in vitro* antioxidant activities.

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Conflict of Interests

The authors have no conflict of interest.

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